

# Identification of $\kappa$ - and $\delta$ -opioid receptor transcripts in immune cells

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**Abstract** To investigate the role of opioids as direct modulators of the immune response, we have searched for expression of the recently cloned  $\delta$ ,  $\mu$  and  $\kappa$  opioid receptors in immune cells. We have devised a reverse transcriptase-polymerase chain reaction strategy which specifically detects a region spanning putative transmembrane regions 2 to 7 for each transcript in both human and mouse immune cells. In human peripheral blood lymphocyte and monocyte preparations,  $\delta$  was undetectable while the  $\kappa$  transcript was present. The analysis of human cell lines revealed low but significant levels of  $\delta$  opioid receptor transcripts in T, B or monocyte cell lines while the  $\kappa$  transcript was found in B cell lines only. Investigation of murine cells showed the presence of transcript for the  $\delta$  receptor in splenocytes and in some T and B cell lines. Unexpectedly, no expression of the  $\mu$  receptor was detected. Sequence analysis of PCR products demonstrated nucleotide identity between immune and neuronal transcripts, indicating that they derive from the same genes. In conclusion, our results lead to the identification of  $\kappa$  and  $\delta$  opioid receptor transcripts in immune cells.

**Key words:** Opioid receptor transcript; Opioid receptor ( $\delta$ ;  $\mu$ ;  $\kappa$ ); Reverse transcriptase-polymerase chain reaction; Immune cell

## 1. Introduction

Opioids are involved in numerous physiological processes including pain control, response to stress, locomotion and cognition, as well as neuroendocrine, autonomic and immune physiology. The opioid system consists of a family of endogenous peptides which act through three classes of membrane receptors [1] referred to as  $\mu$ ,  $\delta$  and  $\kappa$ . These receptors are also targets for exogenous opiate drugs and mediate their strong analgesic and psychotropic actions. The recent molecular cloning of a mouse  $\delta$  opioid receptor from a neuroblastoma cell line [2,3] and the subsequent identification of homologous  $\mu$  [4] and  $\kappa$  [5] cDNAs from rodent brain have demonstrated that opioid receptors in the nervous system belong to the G protein-coupled receptor superfamily with a putative seven transmembrane domain (Tm) topology. The pharmacology of the three cloned receptors correlates well with the previous  $\mu$ ,  $\delta$  and  $\kappa$  classification [6] and their expression pattern parallels binding sites distribution in the nervous system [7].

Evidence for a role of opioids in the modulation of immunity has increased in the last years and multiple reports describe immunosuppressive or immunoenhancing effects of opioids in

both in vivo and in vitro models. It has been suggested that physiological as well as pathological situations which involve the opioid system such as stress, chronic pain treatment and drug addiction might lead to insufficient immune defense against infections or cancer (for reviews see [8–10]). In vivo effects of opioids may be mediated by opioid receptors of the nervous system that would in turn modulate glucocorticoid release, acting therefore indirectly on immune response [11]. Immunomodulation might also result from a direct action of opioids on immune cells, and in vitro studies suggest that immune cells themselves are responsive to opioids. Interestingly, the mode of action of opioids on these cells remains unclear and it has been suggested that it might differ from the opioid pharmacology classically described on neurons. Thus, most reports describe opioid effects on the biological activity of immune cells but few authors demonstrate high-affinity, subtype-specific and stereoselective opioid binding to these cells. Also, several reports have described receptors that do not meet the classical opioid criteria, particularly receptors which bind  $\beta$ -endorphin in a non-naloxone reversible fashion [12–15] or which bind opiate alkaloids, but not opioid peptides, with moderate affinity [16,17].

The cloning of opioid receptors from the nervous system has provided molecular tools to probe opioid function. Therefore, to investigate the possibility that these receptors might be involved in the direct action of opioids on immune response, we have searched for their expression in a wide repertoire of immune cells. This report presents our identification of  $\delta$ - and  $\kappa$ - but not  $\mu$ -opioid receptor transcripts in some of these cells and provides the first molecular evidence for  $\kappa$ -opioid receptor expression in human immune cells and  $\delta$ -opioid receptor expression in murine immune cells.

## 2. Materials and methods

### 2.1. Normal immune cells

Cells from spleen and lymph nodes of C57BL/6  $\times$  SJL mice (6–10 weeks) were obtained as single cell suspensions and depleted from red cells by lysis with  $\text{NH}_4\text{Cl}$ . Murine spleen cells ( $2 \times 10^6/\text{ml}$ ) were cultured in vitro with concanavalin A ( $2 \mu\text{g}/\text{ml}$ , Sigma) in DMEM/10% fetal calf serum (FCS) (Gibco) for 3 days. Human PBL and monocytes from different donors for each cell preparation were depleted from red cells and granulocytes by cytopheresis and elutriation. Human PBL from one preparation (PBL 1) were cultured in vitro with phytohaemagglutinin ( $2 \mu\text{g}/\text{ml}$ , Sigma) in RPMI 1640/10% FCS for 3 days.

### 2.2. Cell lines

SH-SY-5Y, Jurkat, CEM, MOLT-4, EBV and U937 cells were cultured in RPMI-1640 supplemented with 10% FCS. The three EBV cell lines were established from three individuals. HSB2 cells were grown in SMEM supplemented with 10% FCS. EL-4, 11–10, X63-IL-4, CH27 and P388D<sub>1</sub> were cultured in DMEM-10% FCS,  $\beta$ -mercaptoethanol ( $5 \times 10^{-5}$  M), J774 cells in  $\alpha$ 1900 supplemented with 10% FCS and CTLL cells in RPMI-1640 with 10% FCS,  $\beta$ -mercaptoethanol ( $5 \times 10^{-5}$  M) and interleukin 2. All culture media contained 500 U/ml penicillin,

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**Abbreviations:** cDNA, complementary DNA; FCS, fetal calf serum; PBL, peripheral blood lymphocytes; RT-PCR, reverse transcriptase-polymerase chain reaction; Tm, putative transmembrane domain.

100  $\mu$ g/ml streptomycin, 40  $\mu$ g/ml gentamycin and 2 mM glutamine and cultures were maintained at 37°C in 5% CO<sub>2</sub> in a humid atmosphere.

### 2.3. Reverse-transcriptase polymerase-chain-reaction

Before RNA extraction, cells were washed two times with phosphate buffered saline and frozen at –80°C. Total cytoplasmic RNA was isolated by the guanidinium thiocyanate/phenol/chloroform method [18] and RNA integrity was confirmed by agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized from 10  $\mu$ g total RNA at 37°C for 1 h using 200 ng random hexamer oligonucleotide and 400 units of Moloney murine Leukemia Virus reverse transcriptase (BRL) in a reaction volume of 50  $\mu$ l. Correct cDNA synthesis in every sample was controlled by PCR analysis of ribosomal cDNAs (not shown). Opioid receptor transcripts were then amplified using sets of specific primers (Fig. 1) as follows: one tenth of the cDNA was amplified using the *T. aquaticus* polymerase (Taq polymerase, 2 units, Cetus) for forty cycles (1 min 94°C, 1 min 55°C, 1 min 72°C). RT-PCR was performed two to five times for each sample and for each receptor type.

### 2.4. Southern blot analysis

One tenth of the PCR product was resolved by electrophoresis on 1% agarose gel and transferred onto Hybond N<sup>+</sup> nylon membrane (Amersham). PCR products were revealed by hybridization with <sup>32</sup>P-labelled subtype-specific oligonucleotides (Fig. 1) followed by autoradiography. Southern blots were exposed 16 h at –80°C with intensifying screens. Opioid receptor transcripts appeared substantially more abundant in positive controls (brain, neuroblastoma and placenta) and exposure time for these samples was limited to 1.5 h to avoid overexposure.

### 2.5. Cloning and sequencing of the PCR products

PCR products were purified after 1% agarose gel electrophoresis (GeneClean, Bio101), blunt-ended by treatment with DNA polymerase I (Biolabs), and inserted into *EcoRV* sites of the cloning vector pBluescript (Stratagene). Sequencing reactions were performed using the Sanger dideoxy nucleotide chain-termination method (Taq Dye Deoxy terminator Cycle sequencing Kit, Applied Biosystems, Inc.) and analysed on an automated DNA sequencer (373A DNA, Applied Biosystems Inc.) using fluorescently labeled nucleotides.

## 3. Results

### 3.1. PCR strategy

In order to survey expression of the three cloned opioid receptors in murine and human immune cells, we have used a RT-PCR approach. For each receptor type ( $\delta$ ,  $\mu$  and  $\kappa$ ), three specific oligonucleotides (A, B, and C, see Fig. 1) were designed according to previously reported nucleotide sequences [2,3,5,19–22] and on the basis of two requirements: A/B primer pairs span an intron to prevent any possible amplification from traces amounts of genomic DNA possibly present in RNA preparations, and also A, B and C sequences derive from regions which are highly divergent between  $\delta$ ,  $\mu$  and  $\kappa$  receptors to exclude any possible hybridization across subtypes. PCR products were therefore obtained from A/B annealing to specific cDNA molecules and subsequently submitted to Southern analysis, using radiolabelled oligonucleotide C as a probe, to increase the selectivity and the sensitivity of transcript detection. The size of the obtained hybridizing PCR products were as expected from the known cDNA sequences, both in controls and in immune cells. Generally, the signal in immune cells was of considerable lower intensity as compared to that obtained from brain, neuroblastoma or placental positive controls. Results are presented on Fig. 2 for human cells and on Fig. 3 for murine cells.

### 3.2. Expression of opioid receptors in human immune cells

We have analysed normal human immune cell preparations

MOUSE			fragment size
$\delta$	A	5'GAGAGCTCGCGGCCGCCAAGTACTTGATGGAACG3'	697 bp
	B	5'TGGCGGAAGCAGCGCTTGAAGTT3'	
	C	5'CAGCGTCCAGACGATGACGA3'	
$\mu$	A	5'GAGAGCTCGCGGCCGCTACTTGATGGGAACGTGGC3'	785 bp
	B	5'GGAAGCTTGAATTCGGAGGGGTGTTCCCTAGTGT3'	
	C	5'CTGATCACGATTCCAGAAACC3'	
$\kappa$	A	5'GAGAGCTCGCGGCCGCGTCTACTTGATGAATTCCTGG3'	788 bp
	B	5'GGAAGCTTGAATTCGGAAGCAGGATCCTGAAGT3'	
	C	5'GCACCTCCACAGCACAGCTGC3'	
HUMAN			
$\delta$	A	5'GAGAGCTCGCGGCCGCCAAGTACTTGATGGAACG3'	755 bp
	B	5'GGAAGCTTGAATTCCTGAAGCTGTGGGTCTGGGC3'	
	C	5'GGACATCGACCGGCGGACG3'	
$\mu$	A	5'GAGAGCTCGCGGCCGCGTCTACTTGATGAATTCCTGG3'	758 bp
	B	5'GGAAGCTTGAATTCGAGTGGAGTTTGTGCTC3'	
	C	5'GCCTTGGTTACAATCCCCAGA3'	
$\kappa$	A	5'GAGAGCTCGCGGCCGCGTCTACTTGATGAATTCCTGG3'	789 bp
	B	5'GGAAGCTTGAATTCGGTAAGCAGGATCCTGAAGT3'	
	C	5'GCACCTCCACAGCACAGCTGC3'	

ATG

TGA

A

C

B

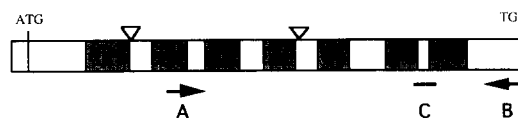


Fig. 1. RT-PCR strategy for the detection opioid receptor mRNA in immune cells. Nucleotide sequence of the primers are derived from published opioid receptor sequences as described in the section 3. Primers A (forward) and B (reverse) have been used for PCR amplification and primers C for hybridization. Expected PCR fragment sizes for each combination are indicated. A scheme of the cDNA is presented below, with putative T<sub>m</sub> domains as grey boxes and splice junctions shown by  $\nabla$ . The localization of the primers A, B and C is shown, relative to the cDNA coding sequence.

for the presence of opioid receptor mRNA, including unstimulated peripheral blood lymphocytes (PBL1, 2 and 3) and monocytes (monocytes 1, 2 and 3) from three individuals. We have detected no mRNA for  $\delta$  or  $\mu$  receptors in these cells. In contrast, one out of three lymphocyte preparations (PBL2) and two out of three monocyte preparations (monocyte 1 and 2) contained significant levels of mRNA for the  $\kappa$  receptor. Using phytohaemagglutinin, we stimulated a PBL preparation which exhibited no detectable opioid receptor expression in the resting state (PBL1). Analysis of this new cell preparation (PBL-PHA) did not show any specific PCR product either, indicating that cell activation did not induce opioid receptor expression. The observation of  $\kappa$  transcript in some but not all preparations suggests possible interindividual variability of  $\kappa$  receptor expression in human lymphocytes and monocytes.

We have analyzed several cell lines derived from tumors, including one monocyte-like cell line (U937), three pre-T cell lines (MOLT-4, CEM, HSB2), one helper T lymphocyte line (Jurkat), and three EBV-transformed B cell lines. All of them, except the Jurkat cell line, showed significant levels of PCR product for the  $\delta$  receptor. The transformed EBV B cell lines display variable levels of transcript for the  $\kappa$  receptor, while all other cell lines are negative. None of the cell lines express detectable levels of mRNA for the  $\mu$  receptor.

We have subcloned and sequenced  $\kappa$  receptor mRNA-derived PCR products obtained from PBL2, monocyte2 and EBV2 cells. Nucleotide sequences were identical to those of

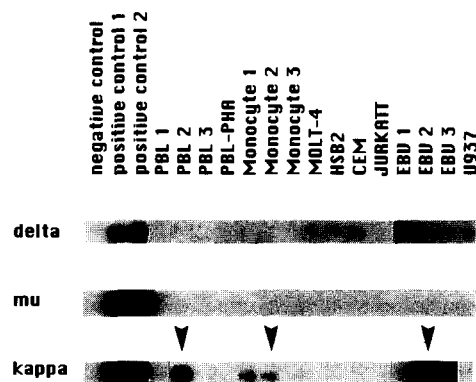


Fig. 2. RT-PCR detection of opioid receptors mRNAs in human immune cells. Positive controls were RNA from human cortex for the three opioid receptor types (positive control 1) or from SH-SY-5Y cells for  $\delta$  and  $\mu$  receptors (positive control 2) and placenta for  $\kappa$  the receptor (positive control 2). In negative controls cDNA was omitted.  $\kappa$  receptor mRNA-derived PCR products indicated by arrows have been cloned, sequenced and found identical to brain-derived PCR products.

opioid receptors cloned from the nervous system (not shown). This result suggests that the human  $\kappa$  receptor cDNA clone previously described in the nervous system and the transcripts detected in the present analysis derive from the same gene.

### 3.3. Expression of opioid receptors in murine immune cells

In mouse, we have analysed spleen cells and lymph node cells which represent normal immune cell populations. We have found a strong RT-PCR signal for the  $\delta$  receptor mRNA from two out of three spleen cell preparations, whereas there was no detectable PCR product for the  $\mu$  and  $\kappa$  receptors from any of these samples.

We have tested the murine T cell lymphoma EL-4, the cytotoxic T cell line CTLL, the T cell hybridomas 11.10 and X63-IL-4, the B cell lymphoma CH27, the B cell hybridoma 25-9-17S and the macrophage cell lines P388D<sub>1</sub> and J774 for the expression of opioid receptors. Significant mRNA levels were found for the  $\delta$  receptor in two T cell lines (EL4 and 11.10) as well as in a B cell line (CH27). No  $\mu$  and  $\kappa$  receptors transcripts were detectable by our method in any of the cell lines analysed.

Analysis of PCR products obtained from spleen 1 and EL-4 RNA samples demonstrates identical nucleotide sequence to that of the mouse neuronal  $\delta$  receptor (not shown). Hence, the  $\delta$  receptor transcript expressed in murine immune cells derives from the same gene as the cloned neuronal receptor.

## 4. Discussion

In this report, we show evidence for the expression of  $\delta$ - and  $\kappa$ -opioid receptor mRNAs in immune cells (See summary in Table 1). Their detection is based upon sequence homology with the recently cloned opioid receptors and the RT-PCR technology. We also provide molecular characterization of the almost entire coding region of these mRNAs, by sequence analysis of PCR products. Nucleotide identity with neuronal cDNAs strongly suggests that they are transcribed from the known  $\delta$ - and  $\kappa$ -opioid receptor genes.

Our study shows that opioid receptor transcripts are not

widely distributed in immune cells. We have investigated a large immune cell repertoire and their occurrence across the various cell types does not necessarily correlate with the opioid pharmacology described in the literature. The absence of the  $\mu$  transcript appears inconsistent with studies reporting the existence of a  $\mu$ -specific pharmacology, such as morphine effects on human lymphocytes [23–25], binding of dihydromorphine on human monocytes [26], [<sup>3</sup>H]BIT (2-(*p*-ethoxy-benzyl)-1-(*N,N*-diethylamino)-ethyl-5-isothiocyanatobenzimidazole) binding or the biological action of morphine and DAMGO on murine splenocyte activity [27,28]. Also, a  $\delta$  receptor has been described in human lymphocytes [29–32] whereas no  $\delta$  mRNA could be detected from human PBL in our study. Similarly, the  $\kappa$ -agonist U50488 was reported to inhibit significantly mouse splenocyte activity [28] while no  $\kappa$  transcript was found in our analysis of murine spleen RNA. Another discrepancy is our finding that none of the cloned opioid receptor is expressed in J774 cells, which have been shown to effectively respond to Dynorphin A in a naloxone-reversible manner [33]. This apparent lack of correspondence might arise from high interindividual variability in humans, as was previously shown for naloxone binding on T lymphocytes [34] or opioid effects on PBL

Table 1

Summary of opioid receptor expression in human and mouse immune cells

	$\delta$	$\mu$	$\kappa$
<b>Human</b>			
Cortex	+++	+++	+++
PBL 1/2/3	-/-/-	-/-/-	-/+/-
PBL-PHA	-	-	-
Monocytes 1/2/3	-/-/-	-/-/-	+/+/-
<b>T cell lines</b>			
MOLT-4	+	-	-
HSB2	+	-	-
CEM	+	-	-
Jurkat	-	-	-
<b>B cell line</b>			
EBV1/2/3	+/+/+	-/-/-	+/+/+
<b>Monocyte line</b>			
U937	+	-	-
<b>Mouse</b>			
Brain	+++	+++	+++
Spleen 1/2/3	++/-/++	-/-/-	-/-/-
Lymph nodes	-	-	-
<b>T cell lines</b>			
EL-4	++	-	-
11.10	+	-	-
CTLL	-	-	-
X63-IL-4	-	-	-
<b>B cell lines</b>			
CH27	++	-	-
25-9-17S	-	-	-
<b>Macrophage lines</b>			
P388D1	-	-	-
J774	-	-	-

RT-PCR signal intensity is indicated as +++: very strong, ++: moderate, +: very low, - undetectable. For human PBL and monocyte preparations, samples from three individuals have been tested, while for mouse spleen cells, three pools of fifteen spleens have been examined.

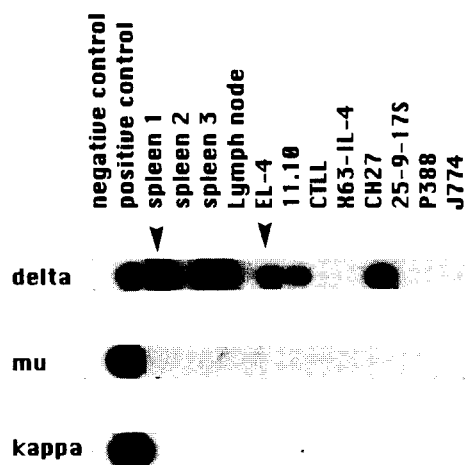


Fig. 3. RT-PCR detection of opioid receptors mRNA expression in mouse immune cells. Positive control was mouse brain RNA for all receptor types and negative controls were performed without cDNA template. Arrows show  $\delta$  receptor mRNA-derived PCR products which have been cloned, sequenced and found identical to brain derived PCR products.

proliferation [35], or be related to strain-dependent immune response in mice [36], or finally be due to the existence of divergent subclones in the model cell lines that we have used. A more likely possibility is that the  $\mu$ ,  $\delta$  and  $\kappa$  opioid effects which have been defined on a pharmacological basis, are mediated by receptors that are different from the cloned receptors.

In line with this hypothesis is also the description of 'non-classical' opioid receptors on immune cells with a pharmacological profile different from the typical  $\mu$ ,  $\delta$  and  $\kappa$  receptors. Particularly,  $\beta$ -endorphin has been shown to interact with human PBL [37], human monocytes [38], U 937 [15], and EL-4 cells [39] in a naloxone non-reversible fashion, thus defining a non-opiate site. This pharmacology is believed to result from the interaction of  $\beta$ -endorphin with a receptor which has not yet been characterized at the molecular level. Interestingly, in these cells, we have identified  $\delta$  or  $\kappa$  mRNAs for the cloned receptors. It would be interesting to investigate further if the putative  $\delta$  and  $\kappa$  mRNA-encoded receptor proteins do participate to  $\beta$ -endorphin binding, possibly in association with other membrane proteins. Another type of unusual opioid pharmacology has been described in human monocytes and J774 cells, as well as in some neuroblastoma cells and the existence of receptors that bind opioid alkaloids but not opioid peptides has been suggested [17]. These receptors, referred to as  $\mu_3$ , would be different from the classically described  $\mu$  receptor and could well be important in mediating opiate drugs action on the immune system. The demonstration of the existence of  $\mu_3$  opioid receptors in J774 cells, and our results showing no  $\mu$ -,  $\delta$ - or  $\kappa$ -opioid receptor transcripts in these cells, again supports the hypothesis of the existence of opioid receptors which derive from genes unrelated to the known  $\mu$ ,  $\delta$  and  $\kappa$  genes.

Some of our findings confirm previously described opioid receptors in immune cells. The observation of significant levels of mRNA encoding the cloned  $\kappa$  receptor human PBL in our study is in accordance with the demonstration that CD4<sup>+</sup> human lymphocytes express  $\kappa$ 1 binding sites [40]. Also we have

found transcripts for the  $\delta$  receptor in mouse spleen RNA, and this finding is consistent with reports that demonstrated the presence of  $\delta$  receptors on mouse cells [41,29] or rat splenocytes [42]. Finally the  $\delta$  transcript found in the MOLT-4 cell line might be responsible for the expression of FITC-naltrexone and [<sup>3</sup>H]superfit (*cis*-(+)-3-methylfentanylisothiocyanate) binding sites previously described on these cells [43].

In normal immune cells, we have not identified transcripts for identical opioid receptor subtypes in mouse and humans. Human PBL express significant levels of  $\kappa$  receptor mRNA while murine splenocytes express  $\delta$  receptor mRNA only. Beyond the species difference, the occurrence of distinct receptor types might originate from the different tissue origin of cell preparations, as exemplified by the report of differential effects of morphine in circulating lymphocytes compared to splenic lymphocytes in rat [44]. Our preparations of normal human immune cells are blood circulating cells while samples for normal murine cells are obtained from the spleen. Our results in mouse and humans are therefore not strictly comparable. One should note however that the  $\delta$  receptor is expressed in many cell lines in both mammals, suggesting that the  $\delta$  receptor is likely to play a role in immune cells independently from the species.

In summary, our study leads to several conclusions: (i) Analysis of opioid receptor expression in a wide variety of cells underscores a possible role for  $\delta$  and  $\kappa$  receptors in immune cells. (ii) The absence of  $\mu$ -opioid receptor transcript and the restricted expression pattern of  $\kappa$  and  $\delta$  receptor, however, does not seem to correlate with the multiple effects of opioids described in the literature. Therefore, the hypothesis that yet uncloned opioid receptors might be responsible for opioid action on immune cells is a reasonable possibility. (iii) Murine T and B cell lines (EL-4 and CH27) as well as human EBV-transformed B cells express substantial levels of  $\delta$  and  $\kappa$  receptors respectively (see Table 1), and may be now used as model cell lines to study the mechanism of action of opioids and the regulation of opioid receptor expression in immune cells at the molecular level. (iv) Our results obtained with normal human immune cells, may now be extended to the analysis of drug-addicted or morphine-treated individuals and allow to enter the physiopathology of opioid receptors in the immune system.

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